

UNITED STATES DEPARTMENT OF AGRICULTURE
AGRICULTURAL RESEARCH ADMINISTRATION

Bureau of Agricultural and Industrial Chemistry
Agricultural Research Center
Beltsville, Maryland

July 17, 1950

TO: G. W. Irving, Jr., Assistant Chief,
BAIC, South Building, Washington, D. C.
FROM: M. W. Kies, Biochemist, Biologically Active
Compounds Div., ARC, Beltsville, Maryland
SUBJECT: Report of trip to Albany, Berkeley and Pasadena, California,
June 20 to July 1, 1950: a) June 21-23 in Albany and Berkeley
to confer with biochemists and plant physiologists at the
Western Regional Research Laboratory and the University of
California; b) June 26-30 in Pasadena to confer with biochemists
and plant physiologists at California Institute of Technology
and to participate in the Conference on Differential Adsorption
at the Fruit and Vegetable Chemistry Laboratory, June 27-29, 1950.

Summary:

The first part of the trip was spent in Albany and Berkeley, during which time I gave a seminar on "A New Multiple Extraction Technique" at the Western Regional Research Laboratory. Conferences with various members of the laboratory staff and scientists at the University of California have been summarized in the report. The discussions were mainly concerned with biochemical techniques which should prove to be useful in a study of the mode of action of natural plant-growth regulators. Considerable interest was shown in our multiple distribution technique which promises to have wide application in the purification and isolation of compounds from plants and other sources.

The second week was spent in Pasadena. On Monday and Friday, I visited the Kerckhoff Laboratories of Biology at California Institute of Technology and discussed work on natural plant-growth regulators. On June 27-29, I attended the Conference on Differential Adsorption at the Fruit and Vegetable Chemistry Laboratory and presented a paper on "The Application of Solvent Distribution to the Purification of Natural Plant-Growth Regulators". Since the conference will be covered in detail in a separate report by Dr. Beavens and associates, only brief mention of certain comments made by the invited speakers will be included in this report.

Wednesday, June 21, 1950

Conference with Dr. David Greenberg, Professor of Biochemistry, University of California. - We discussed their study of transmethylation with radio-active carbon compounds. They have observed the following transformations in liver slices and homogenates: Labilo-methyl \rightarrow formaldehyde \rightarrow formic

acid \rightarrow carbon dioxide. Labelled choline, methionine, serine and glycine all yield labelled formaldehyde and formic acid and all steps are reversible except the last one. Thus the transmethylation picture is considerably more complicated than was originally supposed. The concept of a continuously changing methyl "pool" is reminiscent in some respects of Schoenheimer's dynamic concept of protein metabolism.

Dr. Greenberg's group has not yet observed any positive effects of folic acid or vitamin B₁₂ on transmethylation under their experimental conditions. This may be significant in view of the emphasis placed on their interrelationships at the Federation meetings in Atlantic City last April. However, Dr. Greenberg emphasized that their results are preliminary and involve lack of positive evidence, which may depend on their experimental conditions.

Although this discussion was of general biochemical interest, it is not inconceivable that transmethylation reactions are important in plant metabolism. This has not, to our knowledge, been investigated. There is at present no reason for assuming that transmethylation is concerned with plant-growth regulator activity but the available evidence on either score is so vague that preliminary experiments along this line might be worthwhile.

I was interested in observing the experimental set-up in their laboratory for resolution of amino acids by means of ion exchange resins. They use Dowex 50 resin with no preparation other than to give it a distilled water wash and then centrifugation. The resin is suspended in dilute hydrochloric acid (HCl) and the column packed under slight pressure. Ordinary water-cooled condensers are used for the columns to control the temperature. The flow rate is regulated by constant pressure (mercury trap) and by the fineness of the capillary tip. For amino acids, dilute HCl is used as the eluent.

Their fraction collector was made in their own shop from a slow synchronous motor geared to a disk which was perforated around the outer edge to accommodate shallow containers for the samples. Since the rate of rotation is constant, as well as the flow rate of the eluting fluid, each container catches the same number of drops. This is a very inexpensive, but satisfactory, fraction collector.

Conference with Dr. P. K. Stumpf, Professor of Plant Biochemistry (a newly organized Division), University of California. - We discussed their work on plant enzyme systems. He has demonstrated a plant enzyme which converts glutamine to glutamohydroxamic acid and ammonia in the presence of hydroxylamine. Since hydroxylamine does not occur naturally with the enzyme, this reaction is not the purpose of the enzyme in vivo but it offers a convenient means of studying the enzyme and its properties in vitro. The system requires arsenate for maximum activity but partial activation can be achieved with phosphate in lieu of arsenate. Manganese is also required for optimal activity. (This point is especially interesting in view of the well-known disruption of the

nitrogen metabolism of plants grown in manganese-deficient media.)

Mr. S. Tewfik and Dr. Stumpf showed me certain techniques which they are using in their study of carbohydrate metabolism in plants. Their enzyme preparations are mainly acetone powders prepared by homogenizing the plant parts in cold acetone in a Waring Blender. The plant pigments and other fat-soluble materials are removed by the acetone and the white powder formed is stable indefinitely if kept cold and dry.

Dr. Radin, also in the Division of Plant Biochemistry asked to see me to discuss our automatic distribution apparatus. He had learned of our apparatus indirectly from someone who had attended the Federation meetings in Atlantic City. He indicated that he planned to use the apparatus in his work.

Thursday, June 22, 1950

I presented a seminar at the Western Regional Research Laboratory on the automatic solvent distribution apparatus developed in our laboratory for the fractionation of plant-growth regulator preparations. About 50 people attended the seminar. In connection with my discussion of the theory and design, a demonstration involving the separation of dyes by means of this apparatus was used. The equipment for the demonstration was provided by Mr. Jansen and Dr. Nutting of the Enzyme Research Division. The dyes used were yellow 3 and Red 39 (Food and Drug Cosmetic dyes) and the solvent pair was made up as follows: N-hexane, 5 parts; benzene, 5 parts; methyl alcohol, 8 parts and 0.1 N HCl, 2 parts. With 6 tubes and a volume ratio (total volume of upper/volume per tube) of 5 or 6, the separation of the dyes is clear-cut and illustrates the effectiveness of the apparatus in a convincing manner.

The talk covered in considerable detail the design, theory and use of the apparatus, because several people at the laboratory had indicated that they had need for just such a fractionation procedure. The discussion after the speech included questions by Drs. Schwimmer, Dutton (of the Northern Laboratory) Oleott, Makower, Ward, Jansen and Stitt.

Dr. Stitt's inquiry as to the usefulness of the apparatus was answered by a short (10 min.) resumé of our natural plant-growth regulator work and the use we have made of the solvent distribution method in fractionation and identification of the active component in distillers solubles.

Mr. Jansen commented that they had repeated one of our experiments (the distribution of acetic acid in the solvent pair, ethyl acetate, cyclohexane, ethyl alcohol and water) with their apparatus and had confirmed our results, even though their tubes are much smaller than ours (6 in. long with a capacity of 40 ml.).

Prior to the seminar I had spent some time with Mr. Jansen and Dr. Nutting discussing various solvent pairs which might be of use to them in their problem. The usefulness of the apparatus depends entirely

on the availability of a solvent pair (mutually immiscible solvent mixture) in which the solute will distribute to a reasonable degree. If a material is so polar that it will go entirely into the aqueous phase, it can not be purified or fractionated successfully by this means. The possibility of influencing the solubility of a material in a given system by means of a carrier was discussed. The high molecular weight amino derivatives of fatty acids, manufactured by Armour & Co., were suggested as a possible carrier. They intend to use the method if possible to isolate the phosphate-containing fraction of DFP-inactivated chymotrypsin.

At Dr. Fontaine's request I inquired about the progress of work on the toxicity of 4-chloro-phenoxyacetic acid in Dr. DeEds group. He had nothing further to add at this time to the data already in the Triannual Reports. The material possesses a very low order of oral toxicity. They are waiting for the pathologists report on experimental animals receiving different levels of the compound in their diet. This work is done on contract by Dr. A. J. Cox, Professor of Pathology, Stanford Medical School.

Dr. DeEds said that they intended to investigate soon a recent claim that 4-chlorophenoxyacetic acid affects isolated muscle strips in a manner similar to veratrine. This is of interest because of the publicity the compound has received recently as a possible treatment for poliomyelitis.

Friday, June 23, 1950

Conference with Dr. A. A. Benson of the Chemistry Department, University of California. - Dr. Calvin, head of the photosynthesis work at Berkeley, was in Europe. In his absence, Dr. Benson showed us around the laboratory and spent considerable time discussing their work and allied problems with us. Also present in the group were Drs. Stodola, Dimmler, and Dutton of the Northern Regional Research Laboratory and Professor J. Cason of the Chemistry Department.

The group discussed the current argument between Warburg and Emerson on the efficiency of photosynthesis. Benson said he felt that the two views could be reconciled if one interpreted the data correctly. His explanation is that the high efficiency of photosynthesis observed by Warburg depends on an initial burst of carbon dioxide which occurs only under certain experimental conditions conducive to malic acid decarboxylation. Emerson's experimental conditions are such that this reaction does not occur. In other words, the higher rate of carbon dioxide production observed by Warburg is not caused by photosynthesis but by an independent enzyme system.

During the discussion, Dr. Cason asked about our automatic distribution apparatus. He had heard of it from Dr. Stodola who attended the seminar at the Western Regional Research Laboratory the previous day. He commented immediately on its similarity to a series of continuous liquid-liquid extractions. Dr. Cason told me that he had had occasion some time ago to extract 25 liters of aqueous solution with ether and he considered the possibility of constructing a continuous liquid-liquid extractor for

this purpose. He discussed the matter with Dr. Lepkovsky who had in use an extractor designed for 12 liters of aqueous solution. This extractor was extremely tall (over one-story high) and the rate of flow of lighter solvent was very slow because of the high pressure it had to overcome in order to pass through the system. Dr. Cason suspected that for this reason, perhaps, the extra length of the extraction column defeated its own purpose and he set out to determine experimentally just what height was required to achieve equilibration in the system--acetic acid, ether and water. To his surprise, 6 or 7 inches was sufficient for dispersed ether to become completely saturated with acetic acid in water solution--any excess height did not increase the amount of acetic acid extracted. This fact, the ease with which a solute is distributed between immiscible solvents when one is finely dispersed in the other, has been ignored apparently by many chemists in the design of liquid-liquid extractors. The successful operation of our distribution apparatus depends to a large extent on this one factor.

Monday, June 26, 1950.

Conference with Dr. M. L. Stehsel, Biology Division, California Institute of Technology. - We discussed his work on the isolation of bound indoleacetic ("auxin complex") from fresh corn. The compound which has a molecular weight of about 500 is not acidic but is easily decomposed with alkali to give free indoleacetic acid. The complex gives the color test characteristic of indoleacetic acid but is completely inactive in the avena test. The hydrolyzed material is active, however, and the plant tests closely parallel the color tests on unhydrolyzed fractions. Because of the greater convenience of the color test, he has used it almost entirely in studying the purification of the compound. The conditions of the test are essentially those used by Tang and Bonner, Arch. Biochem. 13, 11 (1947).

In filter paper chromatograms the auxin-complex behaves differently from indoleacetic acid (it is much slower moving) and its distribution between ether and water is very different from the free acid. The bound form is not dissolved in ether to any appreciable extent on continuous liquid-liquid extraction at pH 2 for 16 hours.

Dr. Stehsel wanted to test distillers solubles for the presence of auxin-complex but I felt that the initial experiment should be done in our laboratory since we have spent so much time studying this material as a source of plant-growth regulator. He was very agreeable and cooperative about discussing the details of liberation of indoleacetic acid from the "auxin-complex". We are investigating this matter at the present time to determine whether or not yeast fermentation of corn mash liberates the bound indoleacetic acid present in the mature corn kernels. Since indoleacetic acid exists in fresh corn almost entirely in the bound form it is already obvious from our work that yeast fermentation has liberated a large portion of it. Whether there are any significant amounts of the complex still present in distillers solubles remains to be seen.

Conference with Professor A. Haagen-Smit, one of the discoverers of auxins a and b. - Although the discovery of auxin a and b has been questioned frequently in recent years, Haagen-Smit still is convinced of the existence of compounds distinct from indoleacetic acid or any of its derivatives. Neither he nor others have ever been able to confirm the original work, however.

In Dr. Went's absence, Dr. Haagen-Smit showed me the Phytotron which is famous for its elaborate automatic equipment controlling the temperature, humidity and light source of each section of the building. With these facilities, Dr. Went intends to investigate the optimum temperature, moisture and lighting conditions for the production of various agricultural commodities. One of Dr. Went's preliminary findings is that the total growth achieved by the corn plant is greater, though the rate is somewhat slower, when the night temperature is below that ordinarily encountered in the "corn belt". This result, although a minor observation in itself, is suggestive of the inherent possibilities of such a program of plant research. The geographical distribution and the potential yield of American farm products could be completely changed as a result of such studies. The project apparently was conceived by Dr. Went and the money for construction of the expensive building and equipment obtained entirely by donations, no doubt from people who had been convinced of its importance by Dr. Went himself.

Tuesday, June 27 - Thursday, June 29, 1950.

Conference on Differential Adsorption. - The introductory speech of the conference was given by Dr. Zechmeister of the California Institute of Technology, who is generally considered to be the greatest living exponent of chromatographic techniques. He discussed the historical background of this work, the early observations by Tswett on column chromatography, and the underlying principles of this technique: 1) the adsorbed compound must fit the cavities existing in the adsorption column; and 2) there must be forces present to hold that compound in the cavities. Tswett's most important contribution was his concept of the value of adsorption from a streaming liquid as opposed to batch adsorption under equilibrium conditions. Dr. Zechmeister proposed the following definition for chromatography:--a science which includes all the processes allowing resolution of a mixture on a solid support by means of a liquid phase streaming in a given direction. There are two limitations of the technique as an analytical method: 1) Failure to separate constituents on a column is not a sure proof of homogeneity; 2) there is always a possibility of a chemical reaction occurring on the column.

If no chemical reactions are involved, the classical system then consists of a solute, an adsorbent, and a developer. It is possible to invert the sequence of adsorption and elution of similar compounds by changing either the adsorbent or the developer. Often inversion of sequence may be brought about by merely changing the quantitative composition of a mixed solvent or by raising the temperature of the column.

Dr. Zechmeister discussed Willstätter's classical work on the use of strong adsorbents in the purification of enzymes. Because he used batch

adsorption methods it was not strictly a chromatographic technique. Mild adsorbents in column form may also be used in the purification of enzyme proteins; e.g., emulsin has been fractionated on a bauxite column into β -glucosidase, α -galactosidase and chitinase. If the enzyme remains on the column instead of being eluted, the location of the band may often be determined by extruding the column and brushing the surface with a suitable indicator. For example, amylase may be detected by brushing the column first with starch and, after sufficient incubation, brushing again with iodine to detect areas of digested and undigested starch.

The paper by Dr. Schroeder, also of California Institute of Technology, on the Moore and Stein starch column technique for amino acid analysis consisted almost entirely of a discussion of experimental details.

Trace metals are removed from the freshly prepared column with β -hydroxyquinoline solution.

Separation of amino acid fractions is improved by cooling the column by means of a water jacket.

Creeping of solutions in the tubes of the fraction collector may be prevented by previous treatment of the tubes with G.E. Dry-film #9987, 5% solution in chloroform. (This might be useful for the prevention of creeping in sample vials when solutions are evaporated for dry weight determinations.)

The advantages of the method are: 1) one can determine 18 amino acids and ammonia on one analytical sample; and 2) racemization of the amino acids does not affect the results. The disadvantages are that the method is time-consuming, requires an enormous number of analyses and elaborate calculations, and the initial investment for equipment is high (the photoelectric fraction collector alone costs approximately \$2000).

Dr. H. K. Mitchell discussed one of their recent developments--the chromatopile. They have increased the potential capacity of a single sheet of filter paper for chromatographic work by using a stack of paper circles compressed to form a column. 200 to 800 sheets, 7-12.5 cm. in diameter will accommodate a sample of 0.1 to 5 gm. Usually about 40 lb./sq.in. pressure is applied. The sample occupies 1/2 to 1 cm. of the column with a few fresh sheets placed above it to insure uniform spreading of the solvent.

Thin, acid-washed S&S papers have been found to be superior to Whatman #1 for this technique. Purity of the paper is important, although if the paper is eluted with the developing solution when an analysis is required the difficulty caused by soluble impurities is not so great.

For protein separations, the paper may be pretreated with a non-specific protein to minimize general protein reactions. One can then take advantage of the specific activity groups in the protein being

studied and achieve more nearly true chromatographic conditions. For example, 0.5% albumen may be included in the solution used for developing and chromatographing various enzyme preparations.

Dr. Zeehmeister commented that it was possible to cut filter paper circles with sufficient accuracy to pack a glass column with filter paper in the classical Tswett manner. This requires precision machining facilities. The paper circles are removed very easily from the glass tube a few at a time with an instrument which looks somewhat like a bent ice-pick. This procedure combines certain advantages of the chromatopile with the advantages of the flowing liquid chromatogram and elution of the Tswett column.

Friday, June 30, 1950.

Conferences with members of Dr. J. Bonner's group at California Institute of Technology. - Dr. B. Axelrod of the Enzyme Research Division at Albany, who is temporarily stationed at the Institute, was very helpful in showing me around the laboratories and introducing me to the people engaged in plant growth regulator research. I was able to see Dr. Bonner for a short while on Wednesday and he made arrangements at that time for me to observe some laboratory techniques which may prove helpful in our work. Miss Rosamond S. Baker described the coleoptile cylinder test in detail and I was fortunate in being able to observe her work for some time Friday morning. The method has been described in an article by Bonner in the *Am. J. Bot.* 36, 429 (1949). This is a plant test which could be used for enzyme studies and which requires no special greenhouse equipment, either for raising the plant material or carrying out the tests. (It was interesting to note that in the other two laboratories visited which specialized in plant biochemistry--Dr. Benson's and Dr. Stumpf's--the plants used for most of the experimental work were grown in a sand flat near a window or in a dark room equipped with a safe light.)

Dr. S. G. Wildman, who worked with Bonner until recently but who is now Professor of Botany at U.C.L.A., was in the Biology Building and I had an opportunity to discuss his work with him. Some time ago, Dr. Wildman published an article on the proteins of spinach leaves in which he stated that indoleacetic acid was an intrinsic part of one of the proteins isolated. This was questioned by other workers on the basis that tryptophane, by its conversion to indoleacetic acid, could account for the "intrinsic" growth regulating effect of the protein preparation. I asked Dr. Wildman what his more recent experiments had shown concerning this point and the question of the homogeneity of the protein fraction. He indicated that his views had changed somewhat--although he still believes that the sample is homogenous, he no longer considers the indoleacetic acid to be an integral part of the molecule. It probably occurs as a result of decomposition of the protein.

In further discussion with Dr. Stehsel, I learned that Dr. Rodemann (Michigan State College) had visited there recently and also discussed

natural plant-growth regulators with them. Dr. Redemann is in the group at East Lansing which we visited in April, 1949, to discuss the progress of their work. At that time they felt that they had essentially completed their investigations on corn pollen, but nothing has been published since. I was told that Redemann, meanwhile has isolated a crystalline active compound from corn pollen but is waiting until they are able to confirm its structure by synthesis before they publish their data.

Conclusions and Recommendations:

1. The principal recommendation is that similar conferences (not necessarily on the same subject matter) be held as frequently as is feasible by members of the Bureau staff. The conference held at Pasadena far exceeded our optimistic expectations concerning its value to the participants and to the Bureau as a whole. Many new procedures were introduced to the group and some already published methods were clarified and extended in their usefulness. In connection with the planning of future conferences, it is suggested that at least one-half day be allotted for informal discussions between individuals in addition to the time for a general discussion in which the entire group participates.
2. It is recommended that either Mr. Doukas or Mr. Davis be allowed to spend a few days in Philadelphia conferring with chemists at the Eastern Regional Research Laboratory on techniques of paper chromatography, column chromatography, and ion exchange. I learned from Dr. Porter at the Conference that there was considerable work in progress there which might prove helpful to us in our plant growth regulator isolation work, which is essentially a study of the fractionation of organic acids in crude mixtures. This recommendation is based on the assumption that the above-mentioned procedures would be useful in our work and the most satisfactory means of familiarizing ourselves with the techniques would be to work closely with someone already skilled in the methods.
3. In view of the information obtained at California Institute of Technology on the progress of Dr. Redemann's work on corn pollen growth regulating activity it is suggested that we undertake an investigation of the mode of action of this compound (or compounds) in the stimulation of plant growth. If Redemann and Wittwer publish on the isolation and identification of the compound before we are able to obtain the pure material, we will be in a better position to utilize our experimental resources to study the pure compound. On the other hand, if we continue to concentrate entirely on isolation work and it turns out that they actually have solved the problem satisfactorily, our work will be more or less wasted. For preliminary experiments we could utilize the oat coleoptile cylinder technique of Bonner's or similar work could be done on bean plant parts (which are readily available because of the supply of bean plants maintained for assay purposes).

Marian V. Kies

Action taken on recommendations.

1. I concur in Dr. Kies recommendation that additional conferences on subjects of overall interest to the Bureau should be held.

2. Either Mr. Doukas or Mr. Davis will be sent to the Eastern Regional Research Laboratory to become better acquainted with column chromatography and ion exchange techniques since it appears that these techniques will be useful in their work.

3. After the active compounds present in corn pollen have been further purified or isolated in pure form attention will be given to their mode of action in connection with their application to agricultural problems.

Thomas A. Fontaine

14 - Washington office
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